

# Hypoxia Requires Notch Signaling to Maintain the Undifferentiated Cell State

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## Summary

In addition to controlling a switch to glycolytic metabolism and induction of erythropoiesis and angiogenesis, hypoxia promotes the undifferentiated cell state in various stem and precursor cell populations. Here, we show that the latter process requires Notch signaling. Hypoxia blocks neuronal and myogenic differentiation in a Notch-dependent manner. Hypoxia activates Notch-responsive promoters and increases expression of Notch direct downstream genes. The Notch intracellular domain interacts with HIF-1 $\alpha$ , a global regulator of oxygen homeostasis, and HIF-1 $\alpha$  is recruited to Notch-responsive promoters upon Notch activation under hypoxic conditions. Taken together, these data provide molecular insights into how reduced oxygen levels control the cellular differentiation status and demonstrate a role for Notch in this process.

## Introduction

It is critical for multicellular organisms to maintain oxygen homeostasis, and complex mechanisms have evolved to sense oxygen levels and convert this information into appropriate physiological responses (Bruick, 2003). Hypoxia leads to a change in the energy metabolism that shifts from oxidative phosphorylation to glycolysis. In addition, induction of angiogenesis serves to increase vascular density in an area of localized hypoxia within a tissue, and, in response to systemic hypoxia, erythropoiesis is induced. Hypoxia is also a pathophysiological component of many disorders, including ischemic diseases and cancer (Semenza, 2001).

Cells have developed a molecular signaling mechanism for oxygen sensing. An important mediator is the transcription factor HIF-1 $\alpha$ , which is hydroxylated at specific proline residues by prolyl hydroxylases that act as oxygen sensors (Bruick and McKnight, 2001; Epstein et al., 2001). At normoxia, hydroxylation of HIF-1 $\alpha$  (Ivan et al., 2001; Jaakkola et al., 2001) leads to decreased stability of HIF-1 $\alpha$  through rapid ubiquitylation and degradation by the 26S proteasome. HIF-1 $\alpha$  is ubiquitylated by the E3 ubiquitin ligase pVHL (von Hippel-Lin-

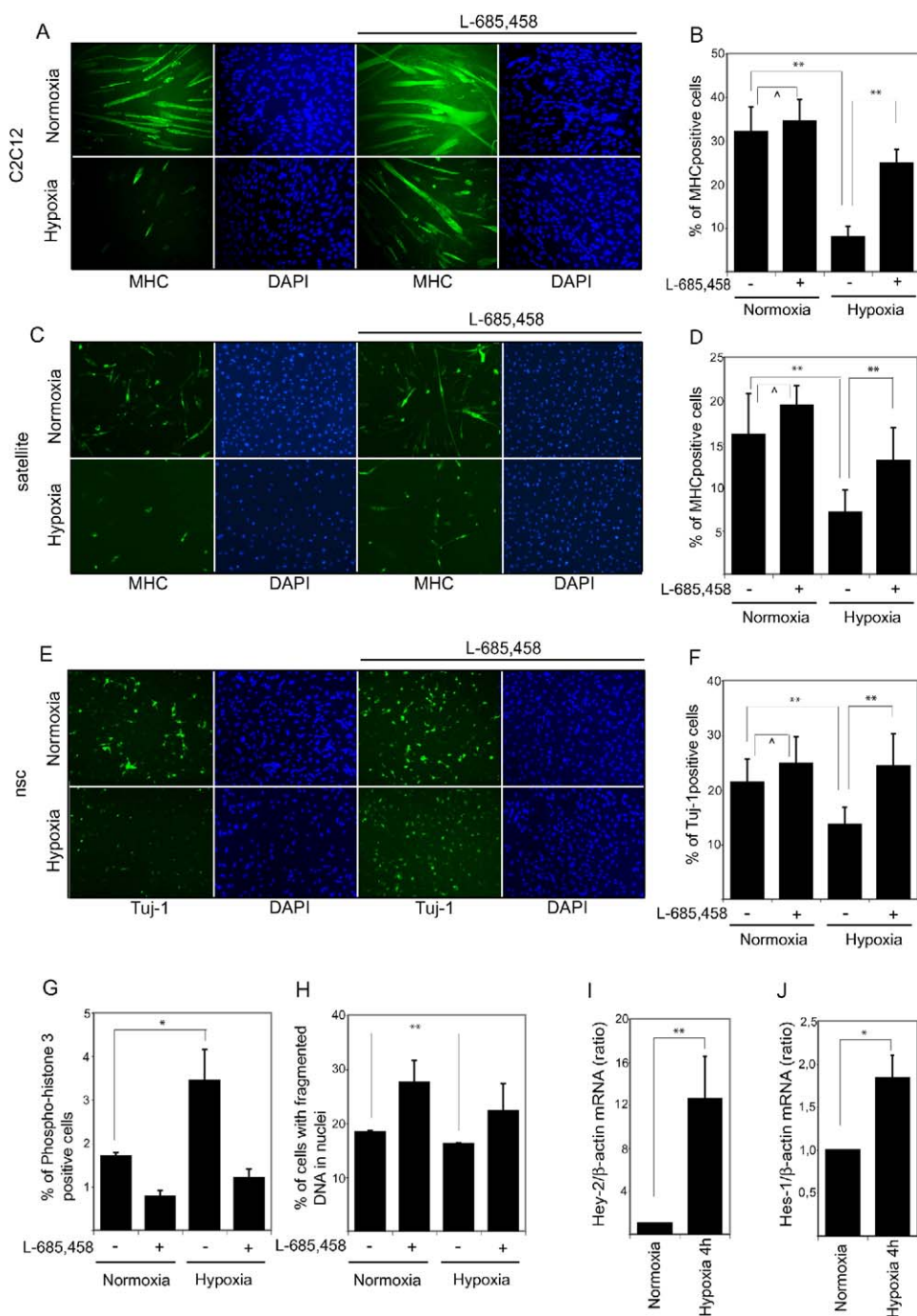
dau tumor suppressor protein) (Cockman et al., 2000; Ohh et al., 2000; Tanimoto et al., 2000). Under hypoxic conditions, HIF-1 $\alpha$  levels are elevated as a result of reduced ubiquitylation and decreased proteasome-mediated degradation. Stabilized HIF-1 $\alpha$  binds, in conjunction with HIF-1 $\beta$  (also referred to as ARNT), to specific hypoxia response elements (HREs) present in the promoters and enhancers of several effector genes. Among the known HIF-1 $\alpha$  target genes are genes important for angiogenesis, erythropoiesis, and glucose metabolism, such as *vascular endothelial growth factor (VEGF)*, *erythropoietin*, and *phosphoglycerate kinase 1 (PGK1)*, respectively (reviewed by Semenza, 2001; Bruck, 2003).

In addition to altering cellular energy metabolism and angiogenesis, hypoxia influences proliferation and differentiation of various stem/progenitor cell populations. This was originally described in neural crest (Morrison et al., 2000) and CNS stem cells (Studer et al., 2000). Culturing of neural crest stem cells at 5% O<sub>2</sub> results in increased proliferation and the formation of more multipotent clones as compared to culturing at 20% O<sub>2</sub> (Morrison et al., 2000). Experimental differentiation of neural crest stem cells under hypoxic conditions allows the cells to differentiate into the sympathoadrenal lineage, a lineage choice not observed in normoxia (Morrison et al., 2000). For CNS precursor cells, culturing at reduced oxygen levels results in increased proliferation and reduced cell death, yielding an increased number of precursor cells. Likewise, differentiation toward the dopaminergic fate was increased (Studer et al., 2000). In addition, hypoxia leads to inhibition of adipocyte differentiation (Yun et al., 2002). As a consequence of these findings, hypoxia is now routinely included in several stem cell culturing protocols to improve yields of stem cells (Rajan et al., 2003). Collectively, these data from different cell types suggest the existence of a more general link between hypoxia and maintenance of the undifferentiated cell state, but little is known about the cellular signaling mechanisms underpinning this process.

Notch signaling is an attractive candidate for this process, as it often functions to maintain the stem/progenitor cell state, for example in myogenesis (Nofziger et al., 1999; Dahlqvist et al., 2003) and hematopoiesis (Varnum-Finney et al., 2000). Notch signaling is evolutionarily highly conserved and critical for controlling cell fate choices in most metazoan species (Artavanis-Tsakonas et al., 1999; Hansson et al., 2004). The Notch receptor undergoes a complex series of at least three different proteolytic cleavages. Activation by DSL (Delta, Serrate, and Lag-2) ligands presented on neighboring cells eventually leads to the liberation of the Notch intracellular domain (Notch ICD), which translocates to the nucleus and interacts with the DNA binding protein CSL (also referred to as RBP-Jk and CBF-1 in mammals, and Suppressor of Hairless [Su(H)] in *Drosophila*). This leads to activation of *Hes* and *Hey* genes (the latter is also known as *Herp*, *Hesr*, *HRT*, *CHF*, and *gridlock*), which, in turn, reduces expression or blocks the function of downstream transcriptional effectors like Neuro-

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**Figure 1. Hypoxia-Induced Inhibition of Myogenic and Neuronal Differentiation Requires Functional Notch Signaling**

(A) Differentiated (MHC-positive) C2C12 cells at normoxia or hypoxia in the presence or absence of  $\gamma$ -secretase inhibitor (L-685,458). (B) Quantification of the percentage of MHC-positive C2C12 cells, relative to DAPI-stained nuclei. “\*\*\*” indicates significant at  $p < 0.01$ , and “^” indicates not significant at  $p > 0.05$  by Student’s *t* test. The bars represent the mean  $\pm$  SE of three independent experiments performed in duplicate. (C) Differentiated (MHC-positive) satellite cells at normoxia or hypoxia in the presence or absence of  $\gamma$ -secretase inhibitor (L-685,458). (D) Quantification of the percentage of MHC-positive satellite cells, relative to DAPI-stained nuclei. (E) Differentiated (Tuj-1-positive) neural stem cells (nsc) treated with hypoxia or kept at normoxia in the presence or absence of  $\gamma$ -secretase inhibitor (L-685,458). (F) Quantification of the percentage of Tuj-1-positive neural stem cells, relative to DAPI-stained nuclei. (G) Proliferative index of neural stem cells (phospho-histone-3 positive) treated with hypoxia or kept at normoxia in the presence or absence of the  $\gamma$ -secretase inhibitor L-685,458. (H) Quantification of the percentage of cells with fragmented DNA in nuclei. (I) Quantification of the ratio of Hey-2/ $\beta$ -actin mRNA. (J) Quantification of the ratio of Hes-1/ $\beta$ -actin mRNA.

genin (Cornell and Eisen, 2002), Mash (de la Pompa et al., 1997), and MyoD (Kopan et al., 1994).

In this report, we asked if Notch signaling plays a role in how hypoxia maintains cells in a more undifferentiated state. We show that hypoxia blocks differentiation of neuronal and myogenic progenitors and that this requires functional Notch signaling. Hypoxia leads to recruitment of HIF-1 $\alpha$  to a Notch-responsive promoter and elevated expression of Notch downstream genes.

## Results

### Hypoxia Inhibits Differentiation in the Myogenic Cell Line C2C12, in Satellite Cells, and in Neural Stem Cells

To test whether hypoxia inhibits differentiation in a cell system that depends on Notch signaling to block differentiation, we first analyzed the myogenic C2C12 cell line. C2C12 cells can be induced to fuse and form myotubes expressing myosin heavy chain (MHC) protein (Soukup et al., 1995), a marker for terminally differentiated myocytes. This process is regulated by Notch, as coculturing with ligand-expressing cells or introduction of the Notch ICD blocks differentiation (Kopan et al., 1994; Kuroda et al., 1999). Culturing of C2C12 cells at hypoxia (1% O<sub>2</sub>) for 4 days resulted in a 4-fold decrease in the number of differentiated cells (32% and 8% MHC-positive cells at normoxia and hypoxia, respectively) (Figures 1A and 1B). To establish whether the inhibition of differentiation was linked to Notch signaling, C2C12 cells were simultaneously treated with hypoxia and a  $\gamma$ -secretase inhibitor (L-685,458), which prevents Notch cleavage and thus the release of Notch ICD from the membrane (Dahlqvist et al., 2003). L-685,458 treatment resulted in a substantial abrogation of the hypoxia-induced inhibition of differentiation, as 25% of the cells were MHC positive (Figures 1A and 1B). This suggests that the hypoxia-induced inhibition of C2C12 differentiation is to a considerable extent dependent on functional Notch signaling. To address whether this Notch dependency is also observed in primary myogenic cells, we tested the same combination of hypoxia and L-685,458 in satellite cells. Reduced differentiation was observed at hypoxia, and differentiation was substantially restored in the presence of L-685,458 (Figures 1C and 1D).

To study whether hypoxia and Notch signaling are also linked in other stem/precursor cell types, we analyzed primary neural stem cells, derived from embryonic rat cortex (Johe et al., 1996; Hermanson et al., 2002). These cells are maintained as neural precursor cells by the presence of fibroblast growth factor-2 (FGF-2) in the culture medium, and they begin to differentiate when FGF-2 is withdrawn (Johe et al., 1996). In keeping with previous reports (Johe et al., 1996), a substantial increase in the proportion of neuronally dif-

ferentiating, i.e., Tuj-1-expressing cells, was observed at 48 hr after FGF-2 withdrawal (22%; Figures 1E and 1F), as compared to 6% when FGF-2 was supplemented to the medium (data not shown). Culture of the cells under hypoxic conditions for 48 hr after FGF-2 withdrawal resulted in a decrease in the number of Tuj-1-positive cells from 22% to 14% (Figures 1E and 1F). The addition of L-685,458 during the 48 hr period completely rescued the inhibitory effect on neuronal differentiation exerted by hypoxia, as 24% of the cells now were Tuj-1 positive (Figures 1E and 1F). The reduction in Tuj-1-positive cells in hypoxia was accompanied by an increase in the number of proliferating cells and a decrease in apoptotic cells (Figures 1G and 1H), whereas the proportion of GFAP-expressing cells was not significantly altered (Figure S1; see the Supplemental Data available with this article online). Taken together, these data establish that the hypoxia-induced effect on blocking neuronal and myogenic differentiation requires functional Notch signaling.

### Hypoxia Increases Expression of Notch Downstream Genes in a Notch-Dependent Manner

As the experiments described above implicated Notch signaling in mediating the effect of hypoxia on differentiation, we next wanted to investigate whether expression of Notch immediate downstream genes (*Hes* and *Hey*) (Greenwald, 1998; Mumm and Kopan, 2000) was altered by hypoxia. We therefore analyzed by real-time PCR *Hes* and *Hey* mRNA levels in C2C12 and neural stem cells. In C2C12 cells, *Hes-1* mRNA levels were not altered by hypoxia (data not shown), whereas *Hey-2* mRNA levels were increased 12-fold after 4 hr of hypoxia treatment (Figure 1I). In neural stem cells, *Hes-1* expression was increased 1.8-fold after 4 hr of hypoxic treatment when compared to cells grown in normoxia (Figure 1J).

To study the relationship between hypoxia and Notch signaling in more detail, we analyzed the hypoxic response in the embryonic carcinoma cell line P19 (McBurney, 1993), which can undergo neuronal differentiation controlled by Notch signaling (Nye et al., 1994). Hypoxia resulted in 4-fold elevated *Hes-1* mRNA expression in P19 cells (Figure 2A). We next analyzed whether the observed increase in *Hes-1* mRNA levels was a result of transcriptional activation. A *Hes-1* reporter construct composed of 0.3 kb of the *Hes-1* promoter linked to the luciferase gene (*Hes-1-luc*) (Jarriault et al., 1995) was transfected into P19 cells. Hypoxia markedly increased expression of the reporter gene, and this increase was largely reduced by L-685,458 (Figure 2B), which suggests that cleavage of the Notch receptor is required. An alternative way to measure Notch-mediated transcriptional activation is to use a synthetic, highly Notch-specific promoter construct. This promoter, 12XCSL-*luc*, is composed of six multi-

(H) Apoptotic index of neural stem cells at normoxia or hypoxia in the presence or absence of the  $\gamma$ -secretase inhibitor (L-685,458).

(I) Quantitative RT-PCR of *Hey-2* mRNA after 4 hr of hypoxia treatment of C2C12 cells.

(J) Quantitative RT-PCR of *Hes-1* mRNA after 4 hr of hypoxia treatment of neural stem cells. Values are significant at \*\* $p < 0.01$  and \* $p < 0.05$ , as indicated in the figure. Bars represent *Hey* or *Hes* mRNA expression relative to expression of  $\beta$ -actin.

Data shown represent the mean  $\pm$  SE of three independent experiments performed in triplicate.

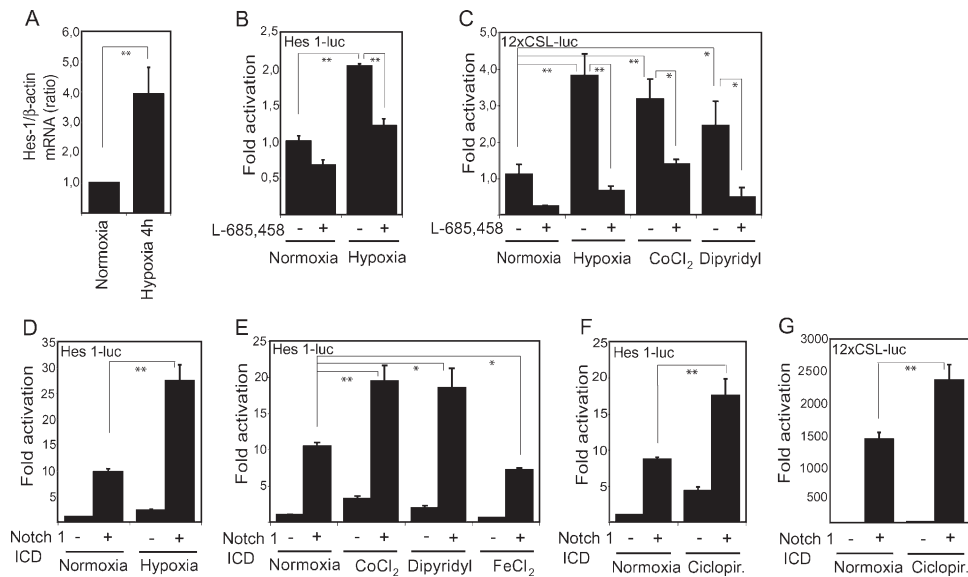


Figure 2. Hypoxia Increases Notch Signaling in P19 Cells

(A) Quantitative RT-PCR of *Hes-1* mRNA in P19 cells cultured at hypoxia for 4 hr. Values are presented as *Hes-1* mRNA expression relative to expression of  $\beta$ -actin. Bars represent the mean  $\pm$  SE of three independent experiments performed in triplicate.

(B) Activation of *Hes-1-luc* in P19 cells treated with hypoxia for 12 hr or kept at normoxia in the presence or absence of  $\gamma$ -secretase inhibitor for 15 hr.

(C) Activation of *12XCSL-luc* after treatment by normoxia, hypoxia,  $\text{CoCl}_2$  (100  $\mu\text{M}$ ), or 2,2'-dipyridyl (100  $\mu\text{M}$ ) for 12 hr in the presence or absence of  $\gamma$ -secretase inhibitor for 15 hr.

(D) *Hes-1-luc* activation after expression of Notch 1 ICD in P19 cells grown at normoxia or hypoxia for 14 hr.

(E) *Hes-1-luc* activation after Notch 1 ICD expression after treatment with  $\text{CoCl}_2$  (100  $\mu\text{M}$ ), 2,2'-dipyridyl (100  $\mu\text{M}$ ), or  $\text{FeCl}_2$  (100  $\mu\text{M}$ ) for 14 hr.

(F) Activation of *Hes-1-luc* by Notch 1 ICD expression in P19 cells grown in normoxia in the presence or absence of cyclopirox olamine (5  $\mu\text{M}$ ) for 14 hr.

(G) Activation of *12XCSL-luc* by Notch 1 ICD during normoxia in the presence or absence of cyclopirox olamine (5  $\mu\text{M}$ ). The graphs represent fold activation relative to  $\beta$ -galactosidase expression. Values are significant at \*\* $p < 0.01$  or \* $p < 0.05$ , as indicated in the figure. The bars represent the mean  $\pm$  SE of two independent experiments performed in triplicate.

merized dimeric CSL binding sites and, thus, responds only to Notch ICD-CSL-mediated activation (Kato et al., 1997). Expression of *12XCSL-luc* was induced by hypoxia (3.9-fold), and this activation was completely blocked by the addition of L-685,458 (Figure 2C). Stabilization and activation of HIF-1 $\alpha$  can be induced in normoxia by agents, which function by chelating iron ions, such as 2,2'-dipyridyl or cyclopirox olamine, or by compounds that substitute ferrous iron with transition cations such as  $\text{Co}^{2+}$  or  $\text{Ni}^{2+}$  (Wanner et al., 2000; Linden et al., 2003). All of these compounds induce HIF-1 $\alpha$  function by inhibiting prolyl and asparaginyl hydroxylase activities involved in HIF- $\alpha$  regulation (Bruick and McKnight, 2001; Epstein et al., 2001; Lando et al., 2002a). The effects on Notch signaling of  $\text{CoCl}_2$  and 2,2'-dipyridyl were tested, and a 2- to 3-fold activation of *12XCSL-luc* was observed (Figure 2C). In sum, these data suggest that hypoxia enhances endogenous Notch signaling, which results in increased activation of transcription of Notch downstream genes.

#### Hypoxia Increases the Activity of Notch 1 ICD

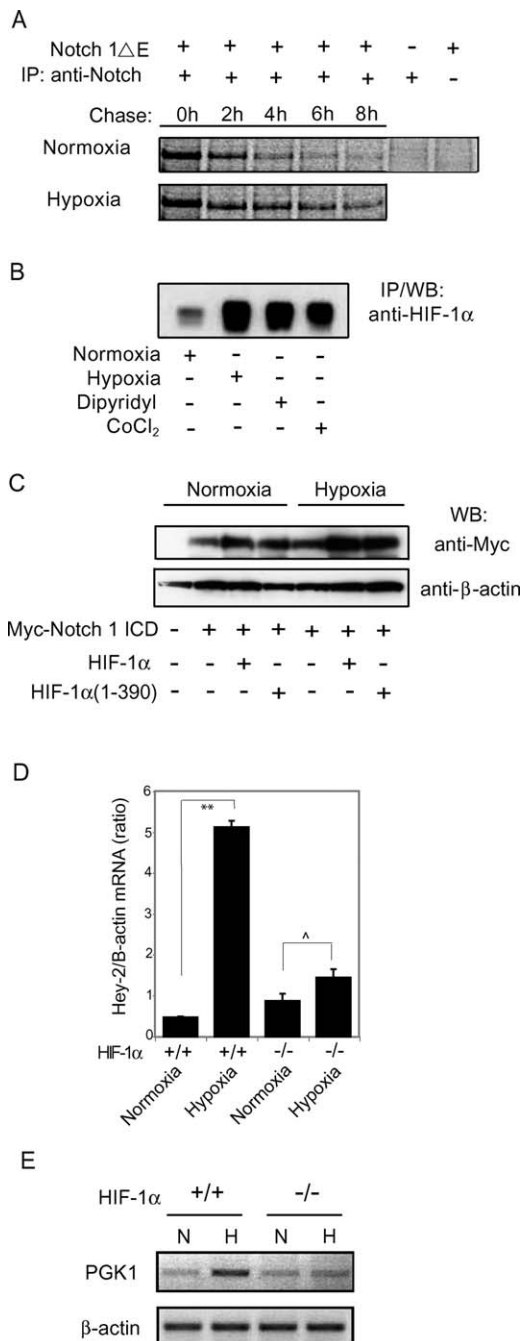
The activation of a Notch response by hypoxia could conceivably involve a number of levels in the Notch signaling system. The  $\gamma$ -secretase inhibitor experiments described above suggest that liberation of Notch ICD is required for the hypoxic response. Transfection of a *Notch 1 ICD* expression plasmid activated the *Hes-1-*

*luc* reporter gene 10-fold in normoxia, consistent with previous reports (Beatus et al., 2001; Blokzijl et al., 2003). This activation was elevated to 27-fold under hypoxic conditions (Figure 2D). When Notch 1 ICD-expressing cells were exposed to  $\text{CoCl}_2$  or 2,2'-dipyridyl, *Hes-1-luc* expression increased to levels similar to those observed in hypoxia-treated cells (approximately 20-fold) (Figure 2E). Moreover, exposure of transfected cells to cyclopirox olamine resulted in elevated expression of both the *Hes-1-luc* and *12XCSL-luc* reporters (Figures 2F and 2G). In the converse experiment, elevation of  $\text{Fe}^{2+}$  levels by addition of  $\text{FeCl}_2$  resulted in reduced *Hes-1-luc* expression, both in cells with endogenous Notch signaling as well as in cells transfected with *Notch 1 ICD* (Figure 2E). The addition of  $\text{FeCl}_2$  has previously been shown to enhance prolyl 4-hydroxylase activity, to enhance interaction of HIF-1 $\alpha$  with pVHL, and, subsequently, to promote degradation of HIF-1 $\alpha$  (Ivan et al., 2001; Jaakkola et al., 2001). Taken together, these data demonstrate that hypoxia and hypoxia-mimicking agents known to regulate HIF function affected signaling mediated by the Notch intracellular domain.

#### Hypoxia Stabilizes the Notch 1 ICD

To test whether the stability of Notch ICD was altered by hypoxia, we measured the protein levels of Notch 1 ICD in P19 cells under normoxic and hypoxic conditions in a pulse-chase experiment. To more closely re-





**Figure 3.** Hypoxia or Ectopically Expressed HIF-1 $\alpha$  Elevates Notch 1 ICD Protein Levels and Increases the Notch Downstream Response

(A) *Notch 1  $\Delta E$*  was transfected into P19 cells and labeled in a pulse-chase experiment with <sup>35</sup>S-methionine. The cells were grown at hypoxia or normoxia and harvested after a chase of indicated length. Notch 1 ICD was immunoprecipitated and analyzed by SDS-PAGE, followed by autoradiography.

(B) Endogenous expression of HIF-1 $\alpha$  in P19 cells grown at normoxia or hypoxia or in the presence of 2,2'-dipyridyl (100  $\mu$ M) or CoCl<sub>2</sub> (100  $\mu$ M) for 8 hr. HIF-1 $\alpha$  was immunoprecipitated, separated by SDS-PAGE, and detected by Western blot by using an anti-HIF-1 $\alpha$  antibody.

(C) Western blot analysis of Myc-Notch 1 ICD protein levels in COS-7 cells at normoxia or hypoxia after expression of Myc-Notch 1 ICD alone or after coexpression with HIF-1 $\alpha$  or HIF-1 $\alpha$ (1-390).

semble the *in vivo* situation, we studied the stability of a Notch ICD generated from a membrane-tethered form of Notch via S3 cleavage, Notch 1  $\Delta E$  (Kopan et al., 1996). Notch 1  $\Delta E$ , which mimics an S2-cleaved receptor, rapidly undergoes S3 processing in the cells, thus generating Notch 1 ICD. Transfection of *Notch 1  $\Delta E$*  was followed by a 4 hr pulse with <sup>35</sup>S-methionine and was chased for 0, 2, 4, 6, and 8 hr under normoxic or hypoxic conditions. Immunoprecipitated Notch 1 ICD showed an increased half-life in hypoxia-treated cells. By 4 hr, approximately 1.8-fold higher Notch protein levels were observed in hypoxia compared to normoxia, a difference that was further elevated to 2.3-fold after 6 hr of chase (Figure 3A). At normoxia, low levels of endogenous HIF-1 $\alpha$  were observed. These were substantially increased by 8 hr of treatment with hypoxia, 2,2'-dipyridyl, or CoCl<sub>2</sub> (Figure 3B).

We next explored whether there was a link between increased amounts of Notch ICD during hypoxia and HIF-1 $\alpha$  or pVHL. First, we transfected COS-7 cells with *Notch 1 ICD* together with *VHL* or *Sel-10*, since *Sel-10* (*cdc4*) is a known negative regulator of Notch ICD protein levels (Oberg et al., 2001). Western blot analysis revealed that *Sel-10*, as expected, reduced the amounts of Notch 1 ICD, while no reduction was observed after the addition of pVHL (data not shown). When *Notch 1 ICD* was transfected together with *HIF-1 $\alpha$*  or a transcriptionally inactive mutant of HIF-1 $\alpha$ , *HIF-1 $\alpha$ (1-390)*, stabilization of Notch 1 ICD was observed, both in normoxia and, more strikingly, in hypoxia (Figure 3C). Collectively, these data show that hypoxia increases the stability of Notch ICD, and they indicate a role for HIF-1 $\alpha$  in this process. To directly investigate whether HIF-1 $\alpha$  is required for the increased Notch activity during hypoxia, we compared the hypoxia-mediated response on *Hey-2* mRNA levels in mouse embryonic fibroblast cells (MEFs) genetically engineered to lack HIF-1 $\alpha$  (Goda et al., 2003). *Hey-2* mRNA levels were robustly increased in wild-type MEFs after 6 hr at hypoxia, while no significant increase was observed in the HIF-1 $\alpha$ <sup>-/-</sup> MEFs (Figure 3D). As expected, *PGK1* mRNA levels were increased during hypoxia only in the wild-type, but not in the HIF-1 $\alpha$ <sup>-/-</sup> MEFs cells (Figure 3E).

#### Notch 1 ICD Interacts with HIF-1 $\alpha$ , but Not with pVHL

In light of the Notch 1 ICD stabilization data, we examined whether Notch ICD physically interacts with HIF-1 $\alpha$  or pVHL. Immunoprecipitation of FLAG-HIF-1 $\alpha$  revealed an interaction with <sup>35</sup>S-labeled Notch 1 ICD, and, conversely, immunoprecipitation of Myc-Notch 1 ICD demonstrated an interaction with <sup>35</sup>S-labeled HIF-1 $\alpha$  (Figure 4A). In contrast, no interaction between Notch 1 ICD and pVHL was observed, whereas, as ex-

(D) Quantitative RT-PCR of *Hey-2* mRNA after 6 hr of hypoxia treatment of wild-type (+/+) or HIF-1 $\alpha$ -deficient (-/-) MEF cells. Values are significant at \*\*p < 0.01 and are not significant at ^p > 0.05, as indicated in the figure. Bars represent *Hey-2* mRNA expression relative to expression of  $\beta$ -actin. Data shown represent the mean  $\pm$  SE of two independent experiments performed in triplicate.

(E) RT-PCR of *PGK1* mRNA in HIF-1 $\alpha$  (+/+) and (-/-) MEF cells cultured at normoxia or hypoxia for 6 hr.

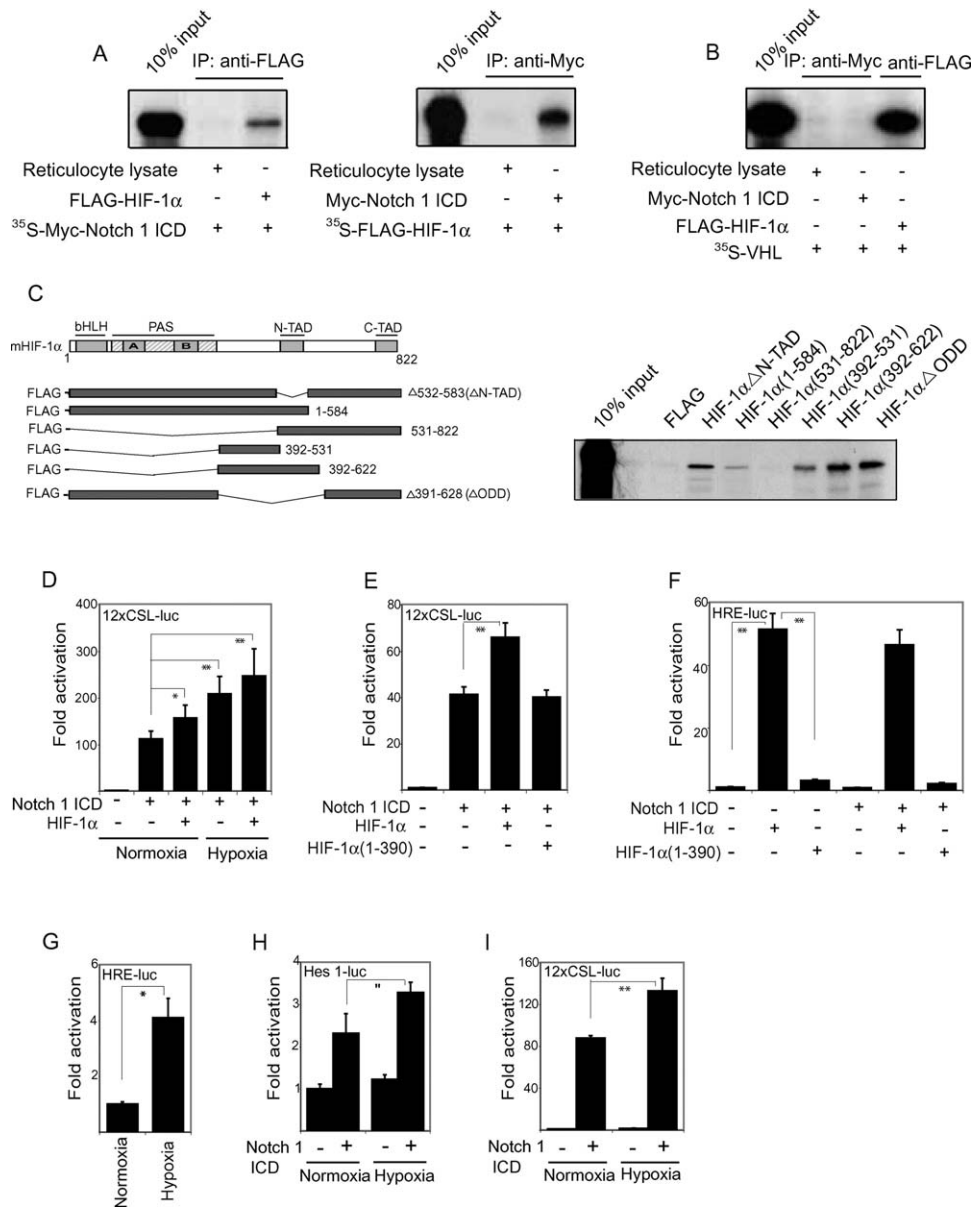


Figure 4. Notch Interacts with HIF-1 $\alpha$ , but Not with pVHL

(A) In vitro-translated FLAG-HIF-1 $\alpha$  and <sup>35</sup>S-Myc-Notch 1 ICD was immunoprecipitated with anti-FLAG (left panel) or FLAG-<sup>35</sup>S-HIF-1 $\alpha$ , and Myc-Notch 1 ICD was immunoprecipitated with anti-Myc (right panel) and subjected to SDS-PAGE and autoradiography.

(B) In vitro-translated <sup>35</sup>S-pVHL, FLAG-HIF-1 $\alpha$ , and Myc-Notch 1 ICD were immunoprecipitated with anti-Myc or anti-FLAG, as indicated and analyzed by SDS-PAGE and autoradiography.

(C) Schematic representation of the several FLAG-tagged HIF-1 $\alpha$  mutants used to map the interaction domain (left). <sup>35</sup>S-Notch ICD and different FLAG-tagged HIF-1 $\alpha$  mutants (as indicated) were immunoprecipitated with anti-FLAG (right panel) and subjected to SDS-PAGE and autoradiography.

(D) Relative luciferase activity after cotransfection of 12XCSL-luc with Notch 1 ICD and HIF-1 $\alpha$  into P19 cells grown at normoxia and hypoxia for 16 hr.

(E) A transactivation dead HIF-1 $\alpha$ , HIF-1 $\alpha$ (1-390), does not enhance Notch ICD-induced activation of 12XCSL-luc at normoxia, whereas wild-type HIF-1 $\alpha$  does.

(F) HIF-1 $\alpha$ , but neither HIF-1 $\alpha$ (1-390) nor Notch 1 ICD, significantly activates transcription of the hypoxia response element (HRE-luc) in P19 cells.

(G) Hypoxia significantly increases the HIF transactivation potential of the HRE-luc reporter gene after cotransfection into A-498 VHL-deficient cells.

(H) Relative luciferase activity after cotransfection of Hes1-luc and Notch 1 ICD in A-498 cells grown at normoxia or hypoxia for 16 hr.

(I) Relative luciferase activity after cotransfection of 12XCSL-luc with Notch 1 ICD in VHL-deficient cells grown at normoxia or hypoxia for 16 hr. Values are presented as fold activation of luciferase expression relative to  $\beta$ -galactosidase expression.

Values are significant at \*\*p < 0.01, \*p < 0.05, and \*p < 0.06, as indicated in the figure. The bars represent the mean  $\pm$  SE of two independent experiments performed in triplicate.

pected (Cockman et al., 2000; Ohh et al., 2000; Tanimoto et al., 2000), HIF-1 $\alpha$  and pVHL interacted (Figure 4B). pVHL did not significantly increase the level of ubiquitylation of Notch 1 ICD, while Sel-10 markedly elevated Notch 1 ICD ubiquitylation (data not shown), in agreement with previous reports (Oberg et al., 2001). Taken together, these observations suggest that the primary link between hypoxia and Notch signaling involves an interaction between Notch ICD and HIF-1 $\alpha$ , but not a direct interaction between Notch ICD and pVHL. To find out which domain(s) in HIF-1 $\alpha$  is responsible for the observed interaction, we generated a set of truncated FLAG-tagged HIF-1 $\alpha$  constructs and tested their potential to interact with Notch 1 ICD. In Figure 4C, we show that a HIF-1 $\alpha$  protein lacking only the N-terminal transactivation domain (N-TAD) and the truncated proteins HIF-1 $\alpha$ (1–584), HIF-1 $\alpha$ (392–531), and HIF-1 $\alpha$ (392–622) (Figure 4C; left panel) interacted with Notch 1 ICD, as did a form of HIF-1 $\alpha$  containing amino acid residues 1–390 linked to 629–822, i.e., lacking residues 391–628 (Figure 4C; right panel). In contrast, the N-terminally truncated HIF-1 $\alpha$ (531–822) showed no interaction with Notch 1 ICD. These data indicate that there is an interaction domain in the N-terminal portion of HIF-1 $\alpha$  spanning amino acid residues 1–390, and a second domain in the region corresponding to amino acid residues 390–531. The C-terminal region of HIF-1 $\alpha$ , from amino acid residue 531 to the C terminus, which is required for transactivation (Ruas et al., 2002; Carrero et al., 2000), appears to be dispensable for interaction with Notch 1 ICD.

#### The C-Terminal Transactivation Domain of HIF-1 $\alpha$ Is Required for Increased Notch Signaling

The protein interaction data suggest a role for HIF-1 $\alpha$  in the stabilization of Notch ICD and increase in Notch signaling. Transfection of HIF-1 $\alpha$  together with Notch 1 ICD into P19 cells resulted in elevated levels of 12XCSL-*luc* expression, both at normoxia and hypoxia (Figure 4D). In contrast, cotransfection of Notch 1 ICD together with HIF-1 $\alpha$ (1–390) did not activate 12XCSL-*luc* (Figure 4E), and similar data were obtained for the other HIF-1 $\alpha$  mutants that interacted with Notch, i.e., HIF-1 $\alpha$ (1–584) and HIF-1 $\alpha$ (392–531) (data not shown). As a control, wild-type HIF-1 $\alpha$ , but not HIF-1 $\alpha$ (1–390), was shown to activate an HRE-*luc* construct (Figure 4F). Conversely, transfected Notch ICD had no detectable effect on HRE-*luc* activation (Figure 4F). In conclusion, these data show that although the HIF-1 $\alpha$  interaction interface with Notch 1 ICD is located in the N-terminal region of HIF-1 $\alpha$ , hypoxia-dependent enhancement of Notch signaling requires the C-terminal region of HIF-1 $\alpha$ .

Next, we investigated the Notch response to hypoxia in the pVHL-deficient cell line A-498 (Gnarra et al., 1994), in which the levels of HIF, in this case HIF-2 $\alpha$ , are elevated both during normoxia and hypoxia (data not shown), but in which HIF is a more potent transcriptional activator of an HRE-*luc* construct during hypoxia (Figure 4G), in keeping with previous reports (Lando et al., 2002b). In this cell line, hypoxia also led to enhanced Notch ICD-induced *Hes-luc* and 12XCSL activities as compared to normoxic conditions (Figures 4H

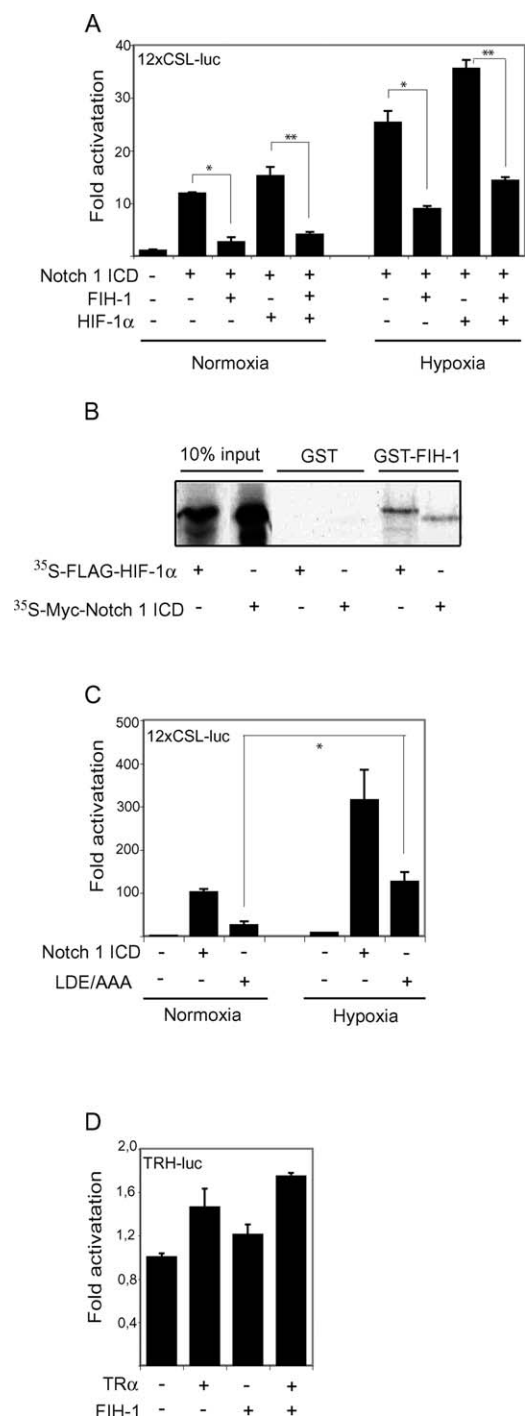
and 4I). Taken together, these data demonstrate that the transactivation domain of HIF-1 $\alpha$  is required for inducing Notch activity, and they further suggest that not only the amount, but also the activity status, of HIF-1 $\alpha$  is important for its ability to activate Notch signaling via Notch ICD.

In the next set of experiments, we investigated the role of FIH-1 (factor inhibiting HIF-1 $\alpha$ ) on activation of the Notch response in hypoxia. FIH-1 reduces the transcriptional activity of HIF-1 $\alpha$  by hydroxylating the asparagine residue 803 of HIF-1 $\alpha$ , which, in turn, blocks HIF coactivator binding (Lando et al., 2002a). As shown in Figure 5A, Notch 1 ICD-induced activation of 12XCSL-*luc* was inhibited by transfection of FIH-1 in P19 cells under either normoxic or hypoxic conditions. Interestingly, cotransfection of FIH-1 significantly reduced activation by Notch 1 ICD also in the absence of transfected HIF-1 $\alpha$  both at hypoxia and normoxia. This suggests that FIH-1 either affects endogenous HIF activity or that FIH-1 plays a more direct role in the regulation of Notch signaling, especially as the HIF-1 $\alpha$  levels are quite low during normoxia (Figure 5A). We also performed protein-protein interaction assays by using bacterially expressed GST-tagged FIH-1 and <sup>35</sup>S-labeled in vitro-translated Notch 1 ICD. In these assays, FIH-1 demonstrated an interaction with Notch 1 ICD, albeit at lower levels than with HIF-1 $\alpha$  (Figure 5B).

As the FIH-1 effect may involve CBP/p300 (Lando et al., 2002a), we tested whether a Notch 1 ICD mutant, which carries a mutation in the CBP/p300 binding site and, thus, is incapable of interacting with CBP/p300 (Notch 1 LDE/AAA) (Oswald et al., 2001), responded to hypoxia. The data show that Notch 1 LDE/AAA is a considerably less potent activator than wild-type Notch 1 ICD, but is still able to activate transcription at hypoxia (Figure 5C). These results indicate that activation of Notch downstream genes by hypoxia is independent of the recruitment of CBP/p300 by Notch 1 ICD, and it will be of interest to learn whether the hydroxylase activity of FIH-1 is required for its effect on Notch. Figure 5D shows that FIH-1 is not a global regulator of gene expression, as induction from a thyroid hormone-responsive promoter (*TRH-luc*) was not significantly altered.

#### HIF-1 $\alpha$ Is Recruited to a Notch-Responsive Promoter in Response to Hypoxia and Activated Notch Signaling

The interaction between HIF-1 $\alpha$  and Notch 1 ICD and the fact that only the transcriptionally active form of HIF-1 $\alpha$  enhanced Notch signaling (Figure 4E) may suggest that HIF-1 $\alpha$  accompanies Notch 1 ICD to a Notch-responsive promoter. This hypothesis was tested by Notch 1 ICD- or HIF-1 $\alpha$ -specific chromatin immunoprecipitation assays (ChIP) under various combinations of hypoxia and Notch activation. Notch activation was achieved in C2C12 cells after coculture with 293T cells stably expressing the Serrate 1 (Jagged 1) ligand, and the cleaved Notch 1 ICD was observed in the C2C12 cells 4 hr after coculture (Figure 6A). When C2C12 cells were subjected to hypoxia, elevated HIF-1 $\alpha$  protein levels were seen 4 hr after the change from normoxic to hypoxic conditions (Figure 6A). In order to monitor chromatin interactions only in the C2C12 cells, not in



**Figure 5. FIH-1 Negatively Regulates Notch Signaling in P19 Cells**  
(A) Coexpression of *12xCSL-luc* and *Notch 1 ICD* with *FIH-1* and/or *HIF-1α* shows that FIH-1 significantly decreases Notch 1 ICD-dependent activation of *12xCSL-luc* both at normoxia and hypoxia. (B) FIH-1 interacts with Notch 1 ICD. In vitro-translated, <sup>35</sup>S-methionine-labeled Notch 1 ICD or HIF-1α were precipitated by bacterially expressed GST-tagged FIH-1 by using glutathione-Sepharose beads. (C) Coexpression of *12xCSL-luc* and *Notch 1 ICD* or *Notch LDE/AAA* during normoxia and hypoxia. Note that the activity of Notch LDE/AAA is increased during hypoxia. (D) Coexpression of *TRH-luc* with *TRα* and/or *FIH-1* in normoxia. Data are presented as fold activation of luciferase expression relative to β-galactosidase expression.

the ligand-carrying 293T cells, we took advantage of the fact that the two cell lines are from different species (mouse versus human) and assigned mouse-specific primers for (1) parts of the promoter of the Notch-responsive gene *Hey-2* (which showed the highest level of induction by hypoxia in C2C12 cells, and which contains a CSL binding site); and (2) for the promoter from the *PGK1* gene (which is part of the canonical hypoxia readout, and which contains HRE sites). **Figure 6A** shows that the *Hey-2* and *PGK1* primers only amplified sequences from mouse (C2C12), not from human (293T), DNA.

The ChIP experiments demonstrated that there were only low levels of Notch ICD bound to the *Hey-2* promoter in non-Notch-activated C2C12 cells, and that these levels were considerably increased when C2C12 cells were cocultured with Serrate 1-expressing 293T cells, both at normoxia and hypoxia (**Figure 6B**, second panel from top). These results are in agreement with recently published observations regarding the *Hes-1* promoter (Fryer et al., 2004). Interestingly, under the combination of hypoxia and ligand-activation of Notch, binding of HIF-1α to the *Hey-2* promoter was observed (**Figure 6B**, top panel). However, HIF-1α was not detected at the *Hey-2* promoter when Notch signaling was not activated. Analysis of the *PGK1* promoter showed that HIF-1α was present at the promoter only in hypoxia, and that the interaction was somewhat elevated when C2C12 cells were cocultured with Serrate 1-expressing cells (**Figure 6B**, third panel from top). In contrast, we did not detect any interaction between Notch 1 ICD and the *PGK1* promoter (**Figure 6B**, lower panel). Finally, we wanted to establish whether Notch signaling affected transcription mediated by the *PGK1* promoter. We observed an increase in the production of *PGK1* mRNA in response to hypoxia in the C2C12 cells, and this increase was slightly reduced in the presence of the γ-secretase inhibitor L-685,458 (**Figure 6C**). In conclusion, these data show that HIF-1α in response to hypoxia and Notch signaling is recruited to a Notch-responsive promoter.

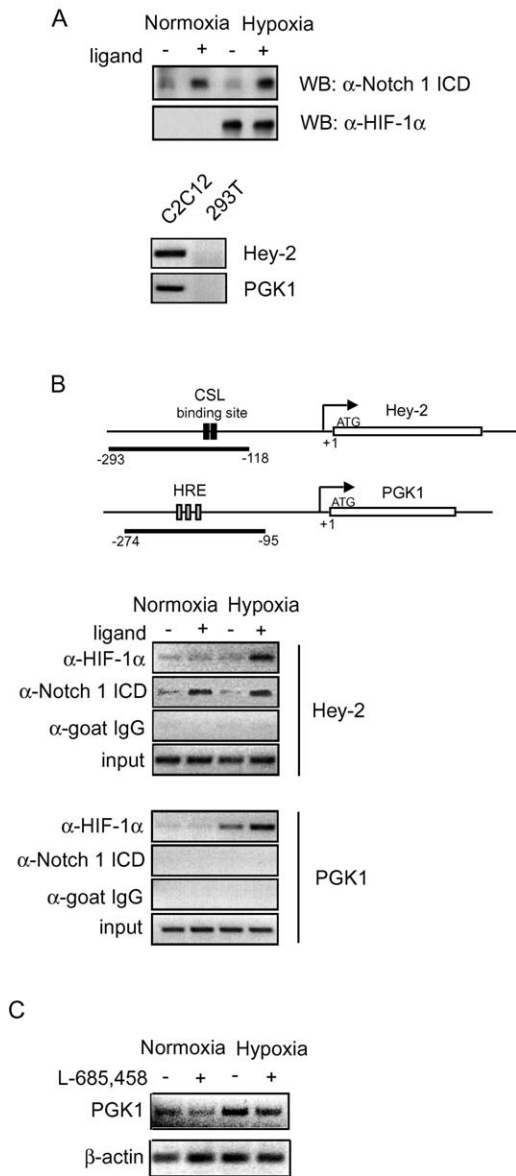
## Discussion

Hypoxia controls many important aspects of cellular life, and a recently discovered function of hypoxia is to regulate differentiation in stem/precursor cells in culture. In CNS precursors, increased proliferation and reduced apoptosis are observed in hypoxia (Studer et al., 2000), while hypoxic neural crest stem cells show promoted survival and proliferation coupled with a broader differentiation potential than in normoxia (Morrison et al., 2000). In this report, we asked whether Notch signaling plays a role in converting the information generated in hypoxia to a signal for maintenance of an undifferentiated cellular state.

We found that hypoxia led to an inhibition of differentiation in cortical neural stem cells, myogenic satellite

Values are significant at \*\*p < 0.01, as indicated in the figure. The bars represent the mean ± SE of two independent experiments performed in triplicate.





**Figure 6.** HIF-1 $\alpha$  Binds to a Notch-Responsive Promoter in Cells Grown at Hypoxia and with Activated Notch Signaling  
(A) Expression levels of Notch 1 ICD and HIF-1 $\alpha$  in C2C12 cells grown at normoxia or hypoxia after coculture with 293T cells stably expressing the Serrate 1 ligand (+) or with control 293T cells (-) for 4 hr. Upper panel: Western blot analysis with anti-HIF-1 $\alpha$  and anti-cleaved Notch antibodies. Lower panel: PCR primers amplifying a part of the *Hey-2* or *PGK1* promoters are specific for mouse DNA (C2C12 cells).  
(B) ChIP analysis of recruitment of Notch 1 ICD and HIF-1 $\alpha$  to the *Hey-2* or *PGK1* promoters. On top, a schematic depiction of the *Hey-2* and *PGK1* promoters with the amplified promoter regions and the positions of the CSL binding site (*Hey-2*) and the HRE sites (*PGK1*) denoted. For the ChIP experiments, C2C12 cells were either cocultured with Serrate 1-expressing 293T cells (+) or with mock 293T cells (-), cultured at normoxia or hypoxia, as indicated. Top panel: immunoprecipitation for HIF-1 $\alpha$  or Notch-1, PCR amplification of the *Hey-2* promoter. Bottom panel: immunoprecipitation for HIF-1 or Notch 1 ICD, PCR amplification of the *PGK1* promoter.  
(C) RT-PCR of *PGK1* mRNA in C2C12 cells cultured at normoxia or hypoxia for 16 hr in the presence or absence of L-685,485.

cells, and C2C12 cells, and that pharmacological blocking of Notch signaling by a  $\gamma$ -secretase inhibitor alleviated this inhibition and restored differentiation under hypoxic conditions. Similarly, we observed activation of direct Notch downstream genes by hypoxia, in agreement with a previous report on upregulation of *Hes-1* mRNA levels in neuroblastoma cells in response to hypoxia (Jogi et al., 2002). Activation of the Notch signaling pathway, which includes stabilization of Notch 1 ICD by hypoxia, appears to be mediated via canonical Notch signaling, i.e., through Notch ICD-CSL interaction, and not to involve direct DNA binding by HIF-1 $\alpha$ , as a synthetic, highly Notch-specific promoter, containing only CSL binding sites and no perfect HIF-1 $\alpha$  responsive elements, was strongly activated under the same conditions.

To our knowledge, the link between Notch signaling and hypoxia represents a novel facet of the hypoxic response. In the canonical hypoxic response, hypoxia acts by altering the stability and activity of HIF-1 $\alpha$ , leading to binding of HIF-1 $\alpha$  to HRE-containing regulatory elements in specific target genes and activation of such genes, e.g., *PAI-1*, *VEGF*, *PGK1*, *transferrin*, and *erythropoietin*. The difference between the canonical hypoxic response and the transfer of hypoxic information into the Notch signaling pathway resulting in the activation of bona fide Notch response genes is schematically depicted in Figure 7.

The present data provide strong evidence for a mechanism of crosstalk between Notch and HIF-1 $\alpha$  signaling pathways, and this mechanism bears an interesting similarity to the recently elucidated mechanism for crosstalk between Notch and BMP/TGF- $\beta$  signaling. In this case, activation of BMP or TGF- $\beta$  signaling leads to activation of Notch through interactions between the intracellular BMP mediator SMAD1 (or SMAD3 for TGF- $\beta$  signaling) with Notch ICD (Blokzijl et al., 2003; Dahlqvist et al., 2003) (reviewed in Kluppel and Wrana, 2005). SMAD1 interacts with Notch ICD and activates *Hes* and *Hey* transcription. In analogy to the enhancement of Notch signaling by hypoxia, BMP signaling is capable of eliciting a response mediated by the *12XCSL-luc* promoter, indicating that there is no need for SMAD DNA binding, as the *12XCSL* promoter is devoid of SMAD binding sites. Similar to the Notch-hypoxia interaction, BMP also blocks myogenic differentiation in C2C12 cells in a Notch-dependent manner (Dahlqvist et al., 2003).

The data presented here indicate that Notch ICD and HIF-1 $\alpha$  are important at the convergence point between the two signaling mechanisms. The importance of Notch ICD is underlined by the ability of  $\gamma$ -secretase inhibitors, which block the S3 cleavage of the Notch receptor and thus liberation of Notch ICD, to strongly reduce the hypoxic response on Notch downstream genes and promoters. Furthermore, the signaling output from an exogenously introduced Notch 1 ICD was modified by hypoxia, leading to increased activation of *12XCSL-luc* and *Hes-luc* in a Notch 1 ICD-dependent manner. The importance of HIF-1 $\alpha$  in this process receives support from the observed interaction between HIF-1 $\alpha$  and Notch 1 ICD, the lack of an hypoxia-induced effect on Notch signaling in fibroblasts devoid of HIF-1 $\alpha$ , and that both the amount and activity status

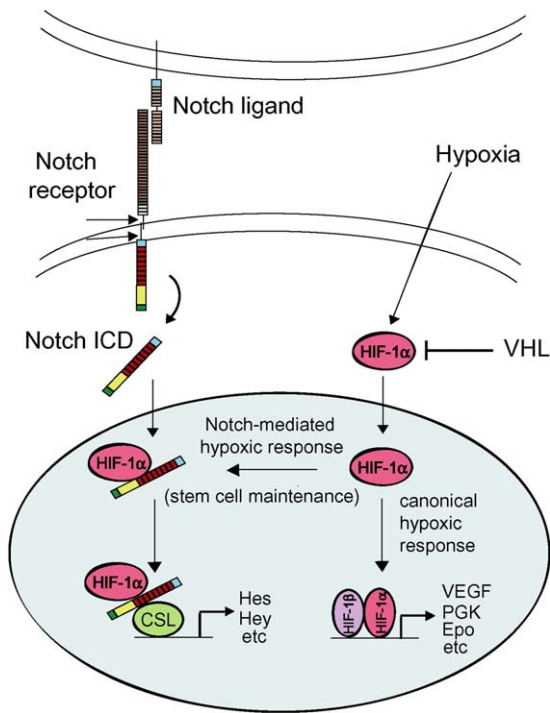


Figure 7. Schematic Depiction of Crosstalk between Notch and Hypoxia

In this model, two modes of the hypoxic response are shown: (1) the canonical hypoxic signaling leading to activation of downstream genes such as *VEGF*, *PGK1*, and *Epo* through HIF1 $\alpha$ /HIF-1 $\beta$  binding to HRE elements in the corresponding promoters; and (2) maintenance of the stem cell state through interaction with the Notch signaling pathway. In the latter case, HIF-1 $\alpha$  and Notch ICD form a point of convergence between the two signaling mechanisms, leading to stabilization of Notch ICD, recruitment of HIF-1 $\alpha$  to Notch-responsive promoters, and activation of Notch downstream genes (*Hey* and *Hes*).

of HIF-1 $\alpha$  correlated with the level of Notch activation. The latter notion is based on the observations that: (1) transfected HIF-1 $\alpha$  elevated the Notch downstream response, (2) a transactivation-inactive form of HIF-1 $\alpha$  left the Notch response unchanged, and (3) the response was augmented in cells lacking VHL. Finally, HIF-1 $\alpha$  was recruited to the *Hey-2* promoter in C2C12 cells in a Notch- and hypoxia-dependent manner. This suggests a mechanism involving an effect of direct transcriptional activation of a Notch-responsive promoter by HIF-1 $\alpha$ , probably as part of a Notch ICD/CSL transcriptional complex (Figure 7). This model is consistent with the observation that a transcriptionally inactive form of HIF-1 $\alpha$ , which was capable of interacting with Notch 1 ICD, did not augment the Notch downstream response. We therefore reason that hypoxia-dependent stabilization of Notch 1CD is not sufficient for activation of the Notch response but may require the recruitment of a form of HIF-1 $\alpha$  containing the C-terminal transactivation domain to the Notch ICD/CSL regulatory complex, possibly potentiating the interaction with transcriptional coactivators. This hypothesis is based on the finding that HIF-1 $\alpha$  is recruited to promoters of Notch downstream genes and the observation that a

mutated form of Notch ICD, unable to interact with CBP/p300, was transcriptionally active at hypoxia. In this context, it will be interesting to learn whether HIF-1 $\beta$  also participates in such a regulatory complex.

The link between hypoxia and Notch described here may have ramifications for other aspects of hypoxia, such as tumor development, in which deregulation of both HIF-1 $\alpha$ - and Notch-mediated signaling events have been implicated (Radtke and Raj, 2003; Weng and Aster, 2004). As many tumors show elevated expression of HIF-1 $\alpha$ , caused by hypoxia inherent to growing tumors and/or genetic loss of VHL (Kondo and Kaelin, 2001), it will be interesting to investigate whether the elevated levels of HIF-1 $\alpha$  are paralleled by increased Notch signaling, and whether the ensuing Notch induction contributes to tumor development.

In conclusion, the data presented here demonstrate a link between hypoxia and Notch signaling and provide insights into how hypoxia maintains the undifferentiated cell state, by using the Notch signaling mechanism. The data also point to an important role for HIF-1 $\alpha$  in this process and to the fact that it can interact with the Notch intracellular domain to link hypoxic information to a Notch response. These data advance our understanding of how Notch crosstalks with other signaling mechanisms and may open up possibilities to control various aspects of the hypoxic response by experimentally manipulating Notch signaling.

## Experimental Procedures

### Plasmid Constructs

*Hes-1 luciferase*, *12XCSL luciferase*, and *TRH-luc* reporter constructs have previously been described (Jarriault et al., 1995; Kato et al., 1997; Balkan et al., 1998). The generation of other constructs used is described in the Supplemental Data available with this article online.

### Cell Culture

Mouse myogenic cell line C2C12 and mouse embryonic teratocarcinoma cell line P19 were grown in Dulbeccos's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS). P19 cells were grown on gelatin-coated dishes. Satellite cells were cultured as previously described (Dahlqvist et al., 2003). mEF cells were cultured as described in Goda et al. (2003). Cortical embryonic stem cells were isolated from rat E14-15 and were cultured in N2 medium. For experimental treatment of cells, see the Supplemental Data.

### Immunocytochemistry

Cells were fixed in 4% paraformaldehyde, washed with PBS, blocked in blocking solution (3% BSA, 1% Triton X-100 in PBS) for 20 min, and incubated with primary antibody for 1 hr. The antibodies used were mouse anti-myosin heavy chain; anti-MHC (MF20, Hybridoma Developmental Studies) diluted 1:15, mouse anti-Tuj-1, recognizing  $\beta$ (III)tubulin (Babco) diluted 1:500, rabbit anti-Phosphohistone 3, recognizing mitotically active cells (Cell Signaling) diluted 1:500, or rabbit anti-GFAP (DakoCytomation) diluted 1:500. After washing, cells were incubated with secondary antibody, goat anti-mouse or goat anti-rabbit Alexa 488 (Molecular Probes), for 40 min before being mounted in ProLong mounting medium (Molecular Probes). Apoptosis was measured by a DNA fragmentation detection kit (Calbiochem). Immunostaining was visualized by using a Nikon Eclipse E800 microscope (20 $\times$  magnification) and digitally imaged with Nikon U-III photographic equipment and the Advanced Spot software package (Version 2.2).

#### RNA Extraction and Quantitative RT-PCR

Cells were grown in normoxic or hypoxic (1% O<sub>2</sub>) conditions for 4 hr. RNA from P19 and C2C12 cells were extracted with Tri Reagent (Sigma), and RNA from embryonic cortical cells was prepared with the Qiagen RNeasy Mini Kit according to the manufacturer's instructions. RNA from fibroblast cells was extracted by the Micro-Midi Total RNA Purification System (Invitrogen) (for details, see the [Supplemental Data](#)).

#### Transfections

Cells were plated on 6-well dishes. P19 and A-498 cells were transfected with Lipofectamine Plus (Life Technologies, Inc.), and COS-7 cells were transfected with FuGene6 Transfection reagent (Roche) according to the manufacturers' instructions (for details, see the [Supplemental Data](#)).

#### Immunoprecipitation Assays

Protein-protein interaction assays were performed by using in vitro-translated proteins produced in rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions, in the absence or presence of <sup>35</sup>S-methionine (for details, see the [Supplemental Data](#)).

#### Protein Extraction and Western Blot

P19 cells were lysed in high-salt buffer (50 mM Tris-HCl, 500 mM NaCl, 1% NP-40, and 20% glycerol) supplemented with 0.5 mM PMSF and 5 mM 2-mercaptoethanol. COS-7 cells were lysed in lysis buffer (10 mM Tris-HCl, 300 mM NaCl, 1.5 mM MgCl<sub>2</sub>, and 0.65% NP-40). Lysates were cleared by centrifugation for 30 min at 14,000 rpm. Western blot analysis is described in the [Supplemental Data](#).

#### Pulse-Chase Experiments

P19 cells transfected with pCS2/Notch 1  $\Delta E$  were cultured in DMEM medium overnight. The next day, culture dishes were pooled and split. A total of 16 hr after transfection, cells were subjected to an <sup>35</sup>S-methionine pulse for 4 hr following a wash with PBS and culture in DMEM in hypoxic conditions (1% O<sub>2</sub>) or in normoxia. Lysis of cells, immunoprecipitation, and gel analysis are described in the [Supplemental Data](#).

#### Coculture and Chromatin Immunoprecipitation Experiments

The system for activation of Notch signaling by coculture has been previously described ([Lindsell et al., 1995](#)). C2C12 cells stably expressing Notch-1 were plated, and the next day either control human 293T or Serrate-1-expressing 293T cells were seeded at a density equal to the monolayer of these cells. The coculture was subjected to either normoxia or hypoxia for 4 hr before isolation of cell extracts. The chromatin immunoprecipitation procedures are described in the [Supplemental Data](#).

#### Supplemental Data

Supplemental Data including data for astrocytic differentiation of neural progenitors and a more detailed description of the Experimental Procedures are available at <http://www.developmentalcell.com/cgi/content/full/9/5/617/DC1/>.

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#### References

- Artavanis-Tsakonas, S., Rand, M.D., and Lake, R.J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* 284, 770–776.
- Balkan, W., Tavianini, M.A., Gkonos, P.J., and Roos, B.A. (1998). Expression of rat thyrotropin-releasing hormone (TRH) gene in TRH-producing tissues of transgenic mice requires sequences located in exon 1. *Endocrinology* 139, 252–259.
- Beatus, P., Lundkvist, J., Oberg, C., Pedersen, K., and Lendahl, U. (2001). The origin of the ankyrin repeat region in Notch intracellular domains is critical for regulation of HES promoter activity. *Mech. Dev.* 104, 3–20.
- Blokzijl, A., Dahlqvist, C., Reissmann, E., Falk, A., Moliner, A., Lendahl, U., and Ibanez, C.F. (2003). Cross-talk between the Notch and TGF- $\beta$  signaling pathways mediated by interaction of the Notch intracellular domain with Smad3. *J. Cell Biol.* 163, 723–728.
- Bruick, R.K. (2003). Oxygen sensing in the hypoxic response pathway: regulation of the hypoxia-inducible transcription factor. *Genes Dev.* 17, 2614–2623.
- Bruick, R.K., and McKnight, S.L. (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* 294, 1337–1340.
- Carrero, P., Okamoto, K., Coumilleau, P., O'Brien, S., Tanaka, H., and Poellinger, L. (2000). Redox-regulated recruitment of the transcriptional coactivators CREB-binding protein and SRC-1 to hypoxia-inducible factor 1  $\alpha$ . *Mol. Cell. Biol.* 20, 402–415.
- Cockman, M.E., Masson, N., Mole, D.R., Jaakkola, P., Chang, G.W., Clifford, S.C., Maher, E.R., Pugh, C.W., Ratcliffe, P.J., and Maxwell, P.H. (2000). Hypoxia inducible factor- $\alpha$  binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. *J. Biol. Chem.* 275, 25733–25741.
- Cornell, R.A., and Eisen, J.S. (2002). Delta/Notch signaling promotes formation of zebrafish neural crest by repressing Neurogenin 1 function. *Development* 129, 2639–2648.
- Dahlqvist, C., Blokzijl, A., Chapman, G., Falk, A., Dannaeus, K., Ibanez, C.F., and Lendahl, U. (2003). Functional Notch signaling is required for BMP4-induced inhibition of myogenic differentiation. *Development* 130, 6089–6099.
- de la Pompa, J.L., Wakeham, A., Correia, K.M., Samper, E., Brown, S., Aguilera, R.J., Nakano, T., Honjo, T., Mak, T.W., Rossant, J., and Conlon, R.A. (1997). Conservation of the Notch signalling pathway in mammalian neurogenesis. *Development* 124, 1139–1148.
- Epstein, A.C., Gleadle, J.M., McNeill, L.A., Hewitson, K.S., O'Rourke, J., Mole, D.R., Mukherji, M., Metzen, E., Wilson, M.I., Dhanda, A., et al. (2001). *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 107, 43–54.
- Fryer, C.J., White, J.B., and Jones, K.A. (2004). Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. *Mol. Cell* 16, 509–520.
- Gnarra, J.R., Tory, K., Weng, Y., Schmidt, L., Wei, M.H., Li, H., Latif, F., Liu, S., Chen, F., and Duh, F.M. (1994). Mutations of the VHL tumour suppressor gene in renal carcinoma. *Nat. Genet.* 7, 85–90.
- Goda, N., Ryan, H.E., Khadivi, B., McNulty, W., Rickert, R.C., and Johnson, R.S. (2003). Hypoxia-inducible factor 1 $\alpha$  is essential for cell cycle arrest during hypoxia. *Mol. Cell. Biol.* 23, 359–369.
- Greenwald, I. (1998). LIN-12/Notch signaling: lessons from worms and flies. *Genes Dev.* 12, 1751–1762.
- Hansson, E.M., Lendahl, U., and Chapman, G. (2004). Notch signaling in development and disease. *Semin. Cancer Biol.* 14, 320–328.
- Hermanson, O., Jepsen, K., and Rosenfeld, M.G. (2002). N-CoR controls differentiation of neural stem cells into astrocytes. *Nature* 419, 934–939.
- Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic,



- A., Asara, J.M., Lane, W.S., and Kaelin, W.G., Jr. (2001). HIF $\alpha$  targeted for VHL-mediated destruction by proline hydroxylation: implications for O<sub>2</sub> sensing. *Science* 292, 464–468.
- Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J., et al. (2001). Targeting of HIF- $\alpha$  to the von Hippel-Lindau ubiquitylation complex by O<sub>2</sub>-regulated prolyl hydroxylation. *Science* 292, 468–472.
- Jarriault, S., Brou, C., Logeat, F., Schroeter, E.H., Kopan, R., and Israel, A. (1995). Signalling downstream of activated mammalian Notch. *Nature* 377, 355–358.
- Jogi, A., Ora, I., Nilsson, H., Lindeheim, A., Makino, Y., Poellinger, L., Axelson, H., and Pahlman, S. (2002). Hypoxia alters gene expression in human neuroblastoma cells toward an immature and neural crest-like phenotype. *Proc. Natl. Acad. Sci. USA* 99, 7021–7026.
- Johe, K.K., Hazel, T.G., Muller, T., Dugich-Djordjevic, M.M., and McKay, R.D. (1996). Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. *Genes Dev.* 10, 3129–3140.
- Kato, H., Taniguchi, Y., Kurooka, H., Minoguchi, S., Sakai, T., Nomura-Okazaki, S., Tamura, K., and Honjo, T. (1997). Involvement of RBP-J in biological functions of mouse Notch1 and its derivatives. *Development* 124, 4133–4141.
- Kluppel, M., and Wrana, J.L. (2005). Turning it up a Notch: cross-talk between TGF  $\beta$  and Notch signaling. *Bioessays* 27, 115–118.
- Kondo, K., and Kaelin, W.G., Jr. (2001). The von Hippel-Lindau tumor suppressor gene. *Exp. Cell Res.* 264, 117–125.
- Kopan, R., Nye, J.S., and Weintraub, H. (1994). The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. *Development* 120, 2385–2396.
- Kopan, R., Schroeter, E.H., Weintraub, H., and Nye, J.S. (1996). Signal transduction by activated mNotch: importance of proteolytic processing and its regulation by the extracellular domain. *Proc. Natl. Acad. Sci. USA* 93, 1683–1688.
- Kuroda, K., Tani, S., Tamura, K., Minoguchi, S., Kurooka, H., and Honjo, T. (1999). Delta-induced Notch signaling mediated by RBP-J inhibits MyoD expression and myogenesis. *J. Biol. Chem.* 274, 7238–7244.
- Lando, D., Peet, D.J., Gorman, J.J., Whelan, D.A., Whitelaw, M.L., and Bruick, R.K. (2002a). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev.* 16, 1466–1471.
- Lando, D., Peet, D.J., Whelan, D.A., Gorman, J.J., and Whitelaw, M.L. (2002b). Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science* 295, 858–861.
- Linden, T., Katschinski, D.M., Eckhardt, K., Scheid, A., Pagel, H., and Wenger, R.H. (2003). The antimycotic ciclopirox olamine induces HIF-1 $\alpha$  stability, VEGF expression, and angiogenesis. *FASEB J.* 17, 761–763.
- Lindsell, C.E., Shawber, C.J., Boulter, J., and Weinmaster, G. (1995). Jagged: a mammalian ligand that activates Notch1. *Cell* 80, 909–917.
- McBurney, M.W. (1993). P19 embryonal carcinoma cells. *Int. J. Dev. Biol.* 37, 135–140.
- Morrison, S.J., Csete, M., Groves, A.K., Melega, W., Wold, B., and Anderson, D.J. (2000). Culture in reduced levels of oxygen promotes clonogenic sympathoadrenal differentiation by isolated neural crest stem cells. *J. Neurosci.* 20, 7370–7376.
- Mumm, J.S., and Kopan, R. (2000). Notch signaling: from the outside in. *Dev. Biol.* 228, 151–165.
- Nofziger, D., Miyamoto, A., Lyons, K.M., and Weinmaster, G. (1999). Notch signaling imposes two distinct blocks in the differentiation of C2C12 myoblasts. *Development* 126, 1689–1702.
- Nye, J.S., Kopan, R., and Axel, R. (1994). An activated Notch suppresses neurogenesis and myogenesis but not gliogenesis in mammalian cells. *Development* 120, 2421–2430.
- Oberg, C., Li, J., Pauley, A., Wolf, E., Gurney, M., and Lendahl, U. (2001). The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian Sel-10 homolog. *J. Biol. Chem.* 276, 35847–35853.
- Ohh, M., Park, C.W., Ivan, M., Hoffman, M.A., Kim, T.Y., Huang, L.E., Pavletich, N., Chau, V., and Kaelin, W.G. (2000). Ubiquitination of hypoxia-inducible factor requires direct binding to the  $\beta$ -domain of the von Hippel-Lindau protein. *Nat. Cell Biol.* 2, 423–427.
- Oswald, F., Tauber, B., Dobner, T., Bourtelee, S., Kostezka, U., Adler, G., Liptay, S., and Schmid, R.M. (2001). p300 acts as a transcriptional coactivator for mammalian Notch-1. *Mol. Cell. Biol.* 21, 7761–7774.
- Radtke, F., and Raj, K. (2003). The role of Notch in tumorigenesis: oncogene or tumour suppressor? *Nat. Rev. Cancer* 3, 756–767.
- Rajan, P., Panchision, D.M., Newell, L.F., and McKay, R.D. (2003). BMPs signal alternately through a SMAD or FRAP-STAT pathway to regulate fate choice in CNS stem cells. *J. Cell Biol.* 161, 911–921.
- Ruas, J.L., Poellinger, L., and Pereira, T. (2002). Functional analysis of hypoxia-inducible factor-1  $\alpha$ -mediated transactivation. Identification of amino acid residues critical for transcriptional activation and/or interaction with CREB-binding protein. *J. Biol. Chem.* 277, 38723–38730.
- Semenza, G.L. (2001). Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. *Trends Mol. Med.* 7, 345–350.
- Soukup, T., Pedrosa-Domellof, F., and Thornell, L.E. (1995). Expression of myosin heavy chain isoforms and myogenesis of intrafusal fibres in rat muscle spindles. *Microsc. Res. Tech.* 30, 390–407.
- Studer, L., Csete, M., Lee, S.H., Kabbani, N., Walikonis, J., Wold, B., and McKay, R. (2000). Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen. *J. Neurosci.* 20, 7377–7383.
- Tanimoto, K., Makino, Y., Pereira, T., and Poellinger, L. (2000). Mechanism of regulation of the hypoxia-inducible factor-1  $\alpha$  by the von Hippel-Lindau tumor suppressor protein. *EMBO J.* 19, 4298–4309.
- Varnum-Finney, B., Xu, L., Brashem-Stein, C., Nourigat, C., Flowers, D., Bakkour, S., Pear, W.S., and Bernstein, I.D. (2000). Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. *Nat. Med.* 6, 1278–1281.
- Wanner, R.M., Spielmann, P., Stroka, D.M., Camenisch, G., Camenisch, I., Scheid, A., Houck, D.R., Bauer, C., Gassmann, M., and Wenger, R.H. (2000). Epolones induce erythropoietin expression via hypoxia-inducible factor-1  $\alpha$  activation. *Blood* 96, 1558–1565.
- Weng, A.P., and Aster, J.C. (2004). Multiple niches for Notch in cancer: context is everything. *Curr. Opin. Genet. Dev.* 14, 48–54.
- Yun, Z., Maecker, H.L., Johnson, R.S., and Giaccia, A.J. (2002). Inhibition of PPAR  $\gamma$  2 gene expression by the HIF-1-regulated gene DEC1/Stra13: a mechanism for regulation of adipogenesis by hypoxia. *Dev. Cell* 2, 331–341.